NUCLEAR FACTOR-KAPPA B EXPRESSION IN ORAL LEUKOPLAKIA AND SQUAMOUS CELL CARCINOMA AN IMMUNOHISTOCHEMICAL STUDY

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CERTIFICATE

This is to certify that this dissertation titled **"NUCLEAR FACTOR-KAPPA B EXPRESSION IN ORAL LEUKOPLAKIA AND SQUAMOUS CELL CARCINOMA AN IMMUNOHISTOCHEMICAL STUDY"** is a bonafide dissertation performed by **P. JEYAPREETHA** under our guidance during her post graduate period between 2008-2011.

This dissertation is submitted to **THE TAMIL NADU DR. M. G. R. MEDICAL UNIVERSITY**, in partial fulfilment for the degree of **MASTER OF DENTAL SURGERY IN ORAL PATHOLOGY AND MICROBIOLOGY, BRANCH VI**. It has not been submitted (partial or full) for the award of any degree or diploma.

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CONTENTS

Oral cancer constitutes the sixth most common cancer worldwide and accounts for approximately 5% of all malignant tumours worldwide.[1] In India and South East Asia, Oral Squamous Cell Carcinoma (OSCC) is the third most common malignancy constituting 50% of all malignant tumours.[1] OSCC is the most common type of oral cancer and is often preceded by or associated with potentially malignant lesions or conditions such as leukoplakia and oral submucous fibrosis.[2]

Oral leukoplakia (OL) is "a white patch or plaque that cannot be characterized clinically or pathologically as any other disease".[1] It is the most common potentially malignant lesion of the oral mucosa. Tobacco and areca nut chewing (alone or in combination such as *paan*) are the habits that are positively associated with oral leukoplakia.[3]

Chewing areca nut containing betel quid without tobacco is an independent risk factor for developing oral cancer. When betel quid with tobacco is consumed with alcohol and smoking the relative risk increases eleven fold. [4]

The malignant transformation rate for leukoplakia ranges from 5-20% and is particularly correlated with the degree of dysplasia.[3] The grading of epithelial dysplasia remains subjective as it relies on cellular atypia and architectural disturbances. It is important to identify markers that could help us to ascertain the

malignant transformation of potentially malignant lesions to target them for aggressive treatment. [5]

NF-κ B is a family of nuclear transcription factors identified by Sen and Baltimore in 1986. It is involved in immune response and comprises of hetero or homo dimers of 5 different subunits NF-κB1, NF-κB2, REL A, REL B, cREL. NF-κB's are transiently activated in response to infection or injury and are aberrantly activated in cancers contributing to their pathogenesis and therapeutic resistance.[6]

NF-κB is bound to inhibitor -kappa B (IκB) and is found in the cytoplasm of cells in an inactive form known as the canonical or classical form of NF-κB. In response to stimulus by growth factors or cytokines, IκB is phosphorylated, ubiquitinated and degraded by proteosome resulting in the activation of NF-κB which translocates to the nucleus and regulates target genes involved in immunoregulation, inflammation, proliferation and apoptosis.[7]

NF-κB is involved in the regulation of cell-cycle and apoptosis, thus playing an important role in cell proliferation and survival. Inappropriate NF-κB activation can mediate oncogenesis and tumor progression.[8] It is known to inhibit apoptosis through the induction of anti-apoptotic proteins, and to suppress the apoptotic potential of chemotherapeutic agents, leading to chemoresistance.[8]

The present study was done to evaluate and compare the expression of NF-κB in formalin fixed paraffin embedded tissues of oral leukoplakia, oral squamous cell carcinoma and normal oral mucosa.

Aim of the study

To assess NF-κB expression in Oral squamous cell carcinoma, leukoplakia and normal buccal mucosa.

Hypothesis (null)

There is no difference in the expression of NF-κB in oral squamous cell carcinoma and leukoplakia when compared with normal buccal mucosa.

Objectives of the study

A. To evaluate the expression of NF-κB in formalin fixed paraffin embedded tissues of normal buccal mucosa by immunohistochemistry.

B. To evaluate the expression of NF-κB in formalin fixed paraffin embedded tissue of oral squamous cell carcinoma from patients by immunohistochemistry.

- With areca chewing habit
- With tobacco chewing habit
- With both areca and tobacco chewing
- With both smoking and chewing

C. To evaluate the expression of NF-κB in formalin fixed paraffin embedded tissue of oral leukoplakia (epithelial dysplasia) by immunohistochemistry.

D. To compare the expression of NF-κB n oral squamous cell carcinoma, epithelial dysplasia and normal buccal mucosa.

Study Design

A retrospective study was conducted in Department of Oral and Maxillofacial Pathology, Ragas Dental College & Hospital, Chennai, using archival paraffin embedded tissues.

Study samples

The study material comprised of 80 formalin fixed, paraffin embedded tissue specimens (archival blocks).

- Forty (n=40) histopathologically confirmed OSCC tissue specimens.
- Twenty (n=20) histopathologically confirmed epithelial dysplasia tissue specimens
- Twenty (n=20) normal buccal mucosa tissue specimens

Study subjects

The study comprised of 3 groups:

Group 1 – (CASES)

Forty tissue blocks from patients diagnosed with OSCC clinically and confirmed histopathologically.

Group 2- (CASES)

Twenty tissue blocks from patients diagnosed with leukoplakia clinically and confirmed histopathologically as epithelial dysplasia.

Group 3- (CONTROLS)

Twenty patients who had clinically normal buccal mucosa, reporting to the outpatient department of oral and maxillofacial

surgery for removal of impacted third molar constituted the control group.

Inclusion Criteria

- They had no habit of smoking, alcohol consumption or chewing areca nut.
- They were apparently healthy with no systemic disorders.
- They were not on any medications for systemic diseases like hypertension, diabetes.

Methodology

1. A detailed case history including age, sex and occupation, past medical and dental history, history of habits, drugs and trauma were recorded.
2. General examination and intraoral examination was done.
3. Biopsy was done in both cases and controls.
4. The tissue taken was immediately transferred to 10% buffered formalin
5. After adequate fixation, tissues were embedded in paraffim.
6. From the paraffim embedded blocks 4 micron thick, sections were cut and used, for routine hematoxylin and eosin (H&E) staining and Immunohistochemical (IHC) staining.
7. This project was approved by Institutional Review Board (IRB) of Ragas Dental College & Hospital, Chennai
8. Patient consent was taken for those patients from whom normal mucosa was obtained.

HEMATOXYLIN & EOSIN STAINING

Reagents

- Harry's hematoxylin
- 1% acid alcohol
- Eosin

Procedure

- The slides were deparaffinised in xylene and hydrated through graded alcohol to water.
- The sections on the slides were flooded with Harry's hematoxylin for 5 minutes.
- The slides were washed in running tap water for 5 minutes.
- The slides were differentiated in 1% acid alcohol for 5 minutes.
- The slides were washed well in running tap water for 5 minutes.
- The tissue sections on the slides were then stained in eosin for 30 seconds.
- The slides were washed in running tap water for 1 minute.
- The slides were then dehydrated through alcohol, cleared, mounted and viewed under light microscope (LM).

IMMUNOHISTOCHEMISTRY (IHC)

Armamentarium

- Microtome
- Autoclave
- Hot air oven
- Slide warmer
- Couplin jars
- Measuring jar
- Weighing machine
- APES coated slides
- Slide carrier
- Aluminium foil
- Micro-pipettes
- Toothed forceps
- Electronic timer
- Beakers
- Rectangular steel tray with glass rods
- Sterile gauze
- Cover-slips
- Light microscope

Reagents used

1. Conc. HCl
2. Laxbro soln
3. APES (3 amino propyl tri ethoxy silane)
4. Acetone
5. Citrate buffer
6. Phosphate Buffer Saline (PBS)
7. 3% H_2O_2
8. Deionized distilled water
9. Haematoxylin
10. Absolute alcohol
11. Xylene

Antibodies used

1. Primary antibody – NF-κB (p50) Rabbit polyclonal antibody (SantaCruz).
2. Secondary antibody – SC-2018 Rabbit Avidin Biotin Complex staining system (SantaCruz).

IHC Procedure

Pretreatment of the slides

- The slides were first washed in tap water for few minutes
- The slides were then soaked in detergent solution for 1 hour
- After 1 hour, each slide was brushed individually using the detergent solution and were transferred to distilled water.
- The slides were washed in two changes of distilled water.
- The slides were washed in autoclaved distilled water.

- The slides were immersed in 1 N HCL (100 ml HCl in 900 ml distilled water) overnight.
- The following day slides were taken out of acid and washed in two changes of autoclaved distilled water.
- All the slides were then transferred to slide trays, wrapped in aluminium foil and baked in hot air oven for 4 hours at 180 degrees centigrade.

APES (3 Amino propyl tri ethoxy silane) coating

Slides first dipped in couplin jar containing acetone for 2 minutes

↓

Dipped in APES for 5 minutes

↓

Dipped in two changes of distilled water for 2 minutes each and slides were left to dry

Preparation of paraffin sections

After the slides were dry, tissue section of 4µ thickness were made in a rotary manual microtome. The ribbons of tissue section were transferred onto the APES coated slide from the tissue float bath such that two tissue bits come on to the slide with a gap in between. One of the tissue sections was labeled positive (P) and the other negative (N).

Procedure

The slides with tissue sections were treated with three changes of xylene to remove paraffin wax. They were put in descending grades of alcohol and then rehydrated with water. Slides were then treated with 3% hydrogen peroxide for 30 minutes to quench endogenous peroxidase activity of cells that would otherwise result in non – specific staining. Then the slides were transferred to citrate buffer and autoclaved for antigen retrieval at 15 lbs pressure for 30 minutes. Then the slides were dipped in 3 changes of distilled water for 5 minutes each. Circles were drawn around the tissues, so that the antibodies added later on do not spread and are restricted to the circle. The tissues were incubated in protein blocking serum for one hour in an enclosed hydrated container. Then the slides were wiped carefully without touching the tissue section to remove excess of blocking serum. The primary antibody, rabbit polyclonal antibody, 1:50 dilution was added to positive tissue on the slide and then to the N, PBS was added. The slides were incubated for one hour. Then the slides were wiped carefully without touching the tissue section to remove excess of antibody and washed with three changes of cold PBS for 5 minutes. Then a drop of biotin conjugated secondary antibody was added on both the sections and the slides were incubated for 30 minutes. Later slides were washed in three changes of cold PBS for 5 minutes in each. The slides were wiped carefully without touching the tissue section to remove excess PBS. Then a drop of avidin biotin enzyme

reagent was added on both the sections and the slides were incubated for 30 minutes. The sections were washed in 3 changes of cold PBS for 5 minutes in each. Then the slides were wiped carefully without touching the tissue section to remove excess PBS. Then a drop of freshly prepared DAB (3'–Diaminobenzidine Tetra Hydrochloride – a substrate chromogen) was added on both sections. Slides were then washed in distilled water to remove excess DAB and counter stained with hematoxylin. The slides were placed in a tray with tap water for bluing. Then the slides were transferred to 70% alcohol, 100% alcohol and one change of xylene. The tissue sections were mounted with Di-n-butyl phthalate. The slides were then observed under the microscope. Throughout the procedure care was taken not to dry the tissues.

Positive Control

A known case of NF-κ B positive oral squamous cell carcinoma specimen tissue were fixed, processed, embedded, sectioned and stained in same manner and used as positive control. One positive control tissue slide was included for each batch of IHC procedure.

IHC PROCEDURE FLOW CHART

APES coated slides with 2 paraffin embedded tissues

↓

Placed in xylene thrice (5 minutes each)

↓

Placed in 100% isopropanol (5 minutes)

↓

Placed in 70% isopropanol (5 minutes)

↓

Washed in distilled water thrice (5 minutes each)

↓

Placed in 3% hydrogen peroxide (20 minutes)

↓

Kept in citrate buffer at pH 6 and autoclaved for antigen retrieval and bench cooled for 40 minutes

↓

Washed in distilled water thrice (5 minutes each)

↓

Protein blocking serum added and incubated for one hour

↓

Primary antibody added to the specimen and incubated for one hour

↓

Washed in PBS thrice (5 minutes each)

↓

Secondary antibody added and incubated in an enclosed hydrated container (30 minutes)

↓

Washed in PBS thrice (5 minutes each)

↓

avidin biotin enzyme reagent added and incubated (30 minutes)

↓

Washed in PBS thrice (5 minutes each)

↓

DAB added and incubated in an enclosed hydrated container

(5 minutes)

↓

Washed in PBS thrice (5 minutes each)

↓

Stained with haematoxylin (20 seconds)

↓

Washed in tap water

↓

Placed in 70% isopropanol (1 minute)

↓

Placed in 90% isopropanol (1 minute)

↓

Placed in 100% isopropanol (1 minute)

↓

Placed in xylene (1 dip)

↓

Slides were mounted using DPX

↓

Slides were observed under the LM and graded

Criteria for evaluation of NF-κ B staining

The following parameters were used to evaluate NF-κ B staining

1. *Tissue localization of stain* – Location of NF-κ B staining either in the epithelium and /or connective tissue was recorded. In the epithelium staining in the basal and /or suprabasal layers was recorded.
2. *Cellular localization of stain* – Nucleus and /or cytoplasm
3. *Intensity of the stain* – In each of the positive case the entire tissue was graded as no stain (0), mild (+), moderate (++), and intense (+++).
4. *Labelling index (LI)* – was calculated by dividing the number of positive cells by the total number of cells counted in the slide and expressed as percentage. A minimum of thousand cells was counted for each slide.

$$LI = \frac{\text{Number of positive cells}}{\text{Total number of cells counted}} \times 100$$

Statistical analysis was done using SPSS ^{TM} software (version 10.0.5). p value ≤ 0.05 was considered to be statistically significant.

- Pearson's Chi-square test was done to compare mean age, the distribution of gender and habits, tissue localization of stain, cellular location, nature of stain, intensity of stain and the percentage of cells stained among the three study groups.
- The inter-observer variability for the intensity of stain was assessed by 2 investigators and their reports were confirmed with kappa statistics.
- The mean labeling index between the groups was analysed by Mann Whitney U test.

STRUCTURE AND MOLECULAR MECHANISM OF NF- κ B

NF-κB described in 1986, by Baltimore. D was characterized initially from B-lymphocytes as a nuclear factor necessary for the transcription of the immunoglobulin κ light chain gene.

Nuclear factor-kappaB (NF-κB) is a collective term that refers to a small class of dimeric transcription factors for a number of genes, including growth factors, angiogenesis modulators, cell-adhesion molecules and antiapoptotic factors.[9] Although most NF-κB proteins promote transcription, some act as inactivating or repressive complexes. The most common dimer known "specifically" as NF-κB, is relatively abundant, controls the expression of numerous genes and exist as an inactive cytoplasmic complex bound to inhibitory proteins of the NF-κB inhibitory (Iκ B) family.[10]

The inactive NF-κB –IκB complex is activated by a variety of stimuli, including proinflammatory cytokines, mitogens, growth factors and stress-inducing agents. The release of NF-κB facilitates its translocation to the nucleus, where it promotes cell survival by initiating the transcription of genes encoding stress-response enzymes, cell-adhesion molecules, proinflammatory cytokines and antiapoptotic proteins.[11]

MEMBERS OF THE FAMILY

NF-κB is a family of signal activated transcription factors comprised of hetero-or homo-dimers from 5 different subunits, NF-κB1, NF-κB2, RELA, c REL and RELB. NF-κBs normally are transiently activated in response to infection or injury, but in cancers are aberrantly activated, regulating a transcriptome of hundreds of genes and corresponding proteosome that promote pathogenesis and therapeutic resistance.[12]

NF-κB1 is transcribed as p105 and processed to p50, NF-κB2 is transcribed as p100 and processed to p52 following phosphorylation and proteosome-dependent degradation of their ankyrin repeat –containing c-termini. RELA, RELB and cREL are synthesized in mature form and often form hetero dimers with NF-κB1 or NF-κB2.[13]

ACTIVATION OF NF-κB

There are two pathways that lead to the activation of NF- κB.

Canonical / Classical pathway.

Non – canonical / alternate pathway.

NF-κB1 is often associated with RELA (or cREL) and bound in the cytoplasm in an inactive form by Inhibitor-kappaB (IκB)- α, β or γ, and known as the canonical, or classical form of NF-κB. This form is activated by various components of pathogens or cytokines such as Tumor Necrosis Factor (TNF) or Interleukin-1 (IL-1), primarily via a trimeric Inhibitor-kappaB Kinase (IKK) comprised of α, β and γ subunits, that phosphorylates IκB, leading to IκB

proteasomal degradation and nuclear translocation and DNA binding of the NF-κB1/RELA heterodimer.[14]

NF-κB2 often forms a heterodimer with RELB, known as the non-canonical form of NF-κB. The non-canonical or alternate pathway is activated by other stimuli such as lymphotoxin B, BAFF and CD40L, via an NF-κB Inducing Kinase (NIK) and IKKα dimeric complex.[15]

Aberrant activation of canonical pathway has been broadly implicated in development of many cancers, consistent with its ubiquitous expression and role in promoting cell survival and growth. Aberrant activation of the non- canonical pathway has been demonstrated in B lymphoid malignancies, as well as other cancers.[16]

INHIBITION OF NF-κB

IκB is a large family of inhibitory molecules that includes IκBα, IκBβ, IκBϵ, IκBγ and Bcl-3. The IκB are characterized by the presence of multiple ankyrin repeats and interact with NF- κB via RHD. The RHD serves several functions; this domain can act as the dimerization and DNA binding domain of this class of proteins as it contains nuclear localization sequence and it is the site for binding of IκB. NF – κB binds to 9 - 10 base pair DNA sites (κB sites) as dimers. The activity of NF - κB is primarily regulated by interaction with inhibitory IκB proteins. [17]

IκB kinase (IKK) is present in the cell cytoplasm as an enzyme with serine-protein-kinase activity that is responsible for

IκBα phosphorylation and that links tumor necrosis factor (TNF) – induced and interleukin-1 (IL-1)-induced kinase cascades to NF-κB activation. It is a large, multisubunit complex with three known components. Two of these polypeptide components, IKKα and IKKβ are catalytic subunits, whereas the third component, IKKα (NEMO), has a regulatory function. In most cells, NF-κB is present as a latent, inactive IκB – bound complex in the cytoplasm.[18] The stimulation by different pathogens and other inducers including viruses. Cytokines lead to the activation of signaling cascades that activates the IκB complex and causes phosphorylation of IκB. The NF–κB is then released and translocated to the nucleus where they bind with target genes and regulate their transcription. [19]

ROLE OF NF-κB IN CELL CYCLE REGULATION

The inactive NF-κB-IκB complex is activated by a variety of stimuli, including pro inflammatory cytokines, nitrogens, growth factors and stress-inducing agents. Pro-inflammatory cytokines produced by macrophages, T cells and other cells exert their actions on target cells by transactivating NFκB (i.e., by initiating the signal cascade).[20] Most cells express receptors for the pro-inflammatory cytokines, IL-1β and TNF-α, and they also contain the IKK complex needed for signal transduction. Cells such as leukocytes, vascular endothelial and smooth muscle cells, cardiomyocytes and fibroblasts therefore respond to proinflammatory cytokines by NF-κB activation.[21]

NF-κB activation induces the expression of pro-inflammatory cytokines in a positive feed-back loop. The NF-κB pathway is used not only by pro-inflammatory cytokines but also by microbial products. In particular, endotoxins of gram-negative bacteria signal through NF-κB. Different stimuli initiates different signal-transduction pathways, which are activated by their respective ligands.[22] NF-κB then translocates to the nucleus where, by binding to κB, it initiates transcription of various cytokines, adhesion molecules, vascular endothelial growth factor etc. These transcription products aid in further proliferation of cells.[23]

NF-κB plays a critical role in cell cycle progression during G_0-to-G_1 phase. Inhibition of NF-κB caused impairment of cell cycle progression, retarded G_1 to S-phase transition. Passage through the restriction point in G1 and transit into S phase requires the sequential activation of cyclin-dependent kinases (CDK4, CDK6, CDK2) in complex with their particular G1 cyclins (D or E). The D family of cyclins (D1, D2, D3) in complex with the catalytic subunits CDK4 and CDK6 are the primary holoenzymes required for entry and passage through G1. NF-κB promotes cell cycle progression by regulating the expression of several genes involved in the cell cycle machinery such as cyclin D1, D2, D3, E and c-myc.[24]

DUAL ROLE OF NF-κB

NF-κB plays a major role as a mediator in inhibition of apoptosis in many cell types. Tumor initiation begins with the

prolonged survival of a cell and so, NF-κB's role has an obvious implication for cancer. An anti-apoptotic role of NF-κB has been linked to T cell lymphoma, osteoclasts, melanoma, pancreatic cancer, bladder cancer and breast cancer. Cell types that display an anti-apoptotic role for NF-κB include B cells, T cells, granulocytes, macrophages, neuronal cells and smooth muscle cells.[25]

NF-κB has been shown to play a pro-apoptotic role in addition to its more common anti-apoptotic role. Different activation pathways of NF-κB may cause the expression of proteins that promote apoptosis (eg: Fas, c-myc, p53, IκBα) or inhibit apoptosis (eg: TRAF2, IAP proteins, Bcl-2 like proteins). NF-κB activation variably controls the regulation of cell cycle proteins (eg: cyclin D1 and CDK2 kinase) and the interaction with various cellular components (eg: p300 and p53) that promote or induce apoptosis.[26]

ROLE OF NF-κB IN CANCER

NF-κB has been implicated in carcinogenesis because of its critical roles in cell survival, cell adhesion, inflammation, differentiation and cell growth. The role of NF-κB in different steps of tumoriogenesis are:

NF-κ B ACTIVATION AND CELL PROLIFERATION

In normal cells, NF-κB becomes activated only after the appropriate stimulation and then, upregulates the transcription of its target genes. In tumor cells, different types of molecular alterations may result in impaired regulation of NF-κB activation. In such

cases, NF-κB loses its transient nature of activation and becomes constitutively activated. This leads to deregulated expression of NF-κB controlled genes.[27]

Several genes that mediate cell proliferation are regulated by NF-κB. These include growth factors such as TNF-α, IL-1β and interleukin-6 (IL-6). Besides growth factors, certain cell cycle-regulatory proteins (eg: the cyclin D1 required for transition of cells from G1 to S phase) are also regulated by NF-κB.[28]

In some cells, PGE2 has been shown to induce proliferation of tumor cells. The synthesis of cyclooxygenase-2 (COX-2), which controls PGE2 production, is also regulated by NF-κB activation. It has also been shown that growth factors such as EGF and platelet-derived growth factor (PDGF) induce proliferation of tumor cells through activation of NF-κB.[29]

ACTIVATION OF NF-κ B PROMOTES SURVIVAL OF TUMOR CELLS

Gene products that negatively regulate apoptosis in tumor cells are controlled by NF-κB activation. These include IAP-1, IAP-2, XIAP, cFLIP, TRAF1, TRAF2 and Bcl-xL. Tumor necrosis factor- alpha can cause programmed cell death (i.e, apoptosis) and this is often paralleled by increased NF-κB activation. Inappropriate activation of NF-κB inhibit apoptosis.[30]

NF-κB may upregulate the mitochondrial antiapoptotic factor Bcl-2, perhaps in a positive feed back loop as Bcl-2 downregulates IκBα, thus increasing NF-κB activation. NF-κB has been linked to anti-apoptotic function in tumors such as T-cell lymphoma, melanoma, pancreatic cancer, bladder cancer and breast cancer and in tumor-related cell types such as B cells, T cells, granulocytes, macrophages, neuronal cells, smooth muscle cells and osteoclasts.[31]

NF-κ B MEDIATES THE INVASION OF TUMOR CELLS

NF-κB regulates many genes involved in the promotion of cancer by clonal expansion, growth, diversification, angiogenesis, adhesion, extravasation, degradation of extracellular matrix, etc. The development of cancer is generally categorized into three stages: tumor initiation, tumor promotion and tumor metastasis. Proteases influence tumor invasiveness (eg: the matrix metalloproteinases and the serine protease urokinase- type plasminogen activator [uPA]) which is regulated by NF-κB.[32]

Matrix metalloproteinases (MMPs) promote growth of cancer cells through the interaction of extracellular matrix (ECM) molecules and integrins, cleaving insulin-like growth factors and shedding transmembrane precursors of growth factors, including transforming growth factor-β (TGF-β). uPA is another critical protease involved in tumor invasion and metastasis. Constitutively active phosphatidylinositol-3 kinase (P13K) controls cell motility by regulating the expression of uPA through the activation of NF-κB. Thus one potential way to block the invasion of tumors is to

target NF-κB so that the activation of genes involved in cancer progression is also blocked.[33]

NF-κ B ACTIVATION AND ANGIOGENESIS

Tumor cells just like normal cells, need oxygen to survive and thus can be a limiting factor to progression of tumors. Vascularization of tumors requires the release of angiogenic growth factors (eg: VEGF, MCP-1) from tumor cells and/or inflammatory cells such as macrophages and neutrophils or in response to pro-inflammatory cytokines. Angiogenesis is critical for tumor progression and this process is dependent on chemokines (eg: MCP-1, IL-8) and growth factors (eg: vascular endothelial growth factor [VEGF]) produced by neutrophils and other inflammatory cells. The production of these angiogenic factors has been shown to be regulated by NF-κB activation.[34,35]

NF-κ B AND METASTASIS

The metastasis of cancer requires the migration of cancerous cells both into and out of the vessel walls that transport them to other parts of the body. The abilty to cross vessel walls is mediated by specific molecules that are expressed in response to a number of signals from inflammatory cells, tumor cells, etc. Among those special molecules are ICAM-1, ELAM-1, and VCAM-1, all of which have been shown to be expressed in response to NF-κB activation.[36]

CONSTITUTIVE ACTIVATION OF NF-κ B IN CANCER

Potential mechanism through which NF-κB could play a role in tumorigenesis involves its constitutive activation. Activation of

NF-κB occurs as it is transported from the cytoplasm to the nucleus upon degradation of the inhibitory subunit. In the nucleus it binds to specific κB sites on the DNA and mediates the expression of a number of genes involved in the cellular response to various stresses. Thus when NF-κB is found persistently in the nucleus, it is referred to as constitutive activation.[37]

While many NF-κB stimuli have been identified, the stimulus responsible for constitutive activation of NF-κB in most cell types is not understood. Cells that express constitutively activated NF-κB are resistant to various chemotherapeutic agents.[38]

CARCINOGENESIS AND NF-κB

Hideki Nakayama, Tetsuro Ikebe, Mahiro Beppu *et al* (2001) used immunohistochemistry to examine the expression of NF-κB and the signaling molecules leading to NF-κB activation in 36 untreated biopsy specimens from patients with squamous cell carcinoma (SCC) and in 15 specimens from patients with epithelial dysplasia of the oral cavity.The results suggest that high expression levels of p65 and IKKα contribute to malignant behaviour and antiapoptotic activity in SCC of the oral squamous epithelium.[44]

S. Chen, A. Fribly, and C. Y. Wang *et al* (2002) used enzyme linked immunosorbent assay and western blot analysis to explore the gene therapy approaches relevant to the inhibition of NF-κB signaling, to determine whether Adv-SR-IκBα inhibited NF-κB activation in Oral Squamous Cell Carcinoma (OSCC) cells. The results of the *in vitro* application of a gene therapy stratergy for

oral cancer treatment confirmed that OSCC cells are accessible to adenovirus-mediated gene transfer and the combination of gene therapy with IκBα and TNF was efficient in the induction of apoptosis and OSCC cells. Also inhibition of NF-κB by IκBα gene transfer sensitizes OSCC cells to TNF killing.[39]

Asha Nair, Manickam Venkatraman, Tessy T Maliekal *et al* (2003) have used several techniques such as immunohistochemistry, Western blotting, Semiquantitative Reverse Transcriptase – Polymerase Chain Reaction and Electrophoretic mobility shift assays, and provided evidences for the constitutive activation of NF-κB during cervical cancer progression using 106 paraffin-embedded cervical tissue specimens of different histological grades. In normal cervical tissue and low grade squamous intraepithelial lesions, p50, RelA and IκB-α were mainly localized in the cytosol, whereas in high-grade lesions and squamous cell carcinomas, p50-RelA heterodimers translocated into the nucleus with a concurrent decrease in IκB-α protein.[41]

Lan Li, Bharat B. Aggarwal, Shishir Shishodia *et al* (2004) studied the ability of curcumin (diferuloylmethane), an agent that is pharmacologically safe in humans, to modulate NF-κB activity. Nuclear factor kappa B (NF-κB) has been determined to play a role in cell survival or proliferation in pancreatic carcinoma. 5-human pancreatic carcinoma cell lines were examined using electrophoretic mobility gel-shift assay and immunoblot analysis.

Curcumin down-regulated NF-κB and growth control molecules induced by NF-κB in human pancreatic cells. These effects were accompanied by marked growth inhibition and apoptosis. Through these findings, the authors provided a biologic rationale for the treatment of patients with pancreatic carcinoma using this nontoxic phytochemical.[48]

Guido M. Sclabas, Tadashi Uwagawa, Christian Schmidt *et al* (2005) studied the effects of aspirin on pancreatic carcinoma prevention and to reveal a possible mechanism of aspirin-mediated cancer chemoprevention. An orthotopic mouse model with human pancreatic carcinoma cell lines PANC-1, PANC-1/Puro, and PANC-1/IκBαM was used to study the inhibitory effects of aspirin on pancreatic tumor formation. It was concluded that aspirin repressed tumor formation by PANC-1cells *in vivo* in a prophylactic setting, suggesting a possible mechanism for aspirin's preventive effect in pancreatic carcinoma through inhibition of NF-κB activation and a mechanistic link between inflammation and tumorigenesis.[47]

An Immunohistochemical study by **Alok Mishra, Alok Bharti, Bhuder Das C *et al* (2006)** was done to analyze the activation of NF – κB and its alterations, in the expression of different NF – κB proteins during oral carcinogenesis *in vivo* using tissue biopsies from precancerous and cancerous oral lesions. The role of high risk human papilloma virus (HPV – 16) which activates the p50 / p65 NF – κB complex formation, which promotes differentiation of oral neoplastic cells leading to better prognosis of

the oral cancer was also analysed. Here the total biopsy specimens were 110,out of which 10 cases were normal (n = 10), 34 cases was oral precancer (n = 34) and 66 cases was cancer (n= 66).Expression of p50 protein was 1000 in all the normal tissues and 40% of precancerous lesion & 70% of oral cancers showed moderate to high expression of NF – κB.[1]

Ximing Tang, Diane Liu, Shishir Shishodia *et al* (2006) showed Immunohistochemical expression of NF-κB p65 in 394 lung cancers (370 non-small cell lung carcinomas [NSCLC]; and 24 small cell lung carcinomas[SCLC]) and 269 lung normal epithelium and preneoplastic lesions, including hyperplasias, squamous metaplasias, dysplasias, and atypical adenomatous hyperplasias. High levels of nuclear Immunohistochemical expression of NF-κB p65 were detected in the lung cancers, with significantly higher levels in SCLCs compared with NSCLCs ($P < 0.0001$).

The findings indicate that NF-κB activation plays an important role in lung cancer pathogenesis and NF-κB p65 nuclear expression is an early and frequent phenomenon in the pathogenesis of lung cancer.[42]

Ming Yu, Jason Yeh and Carter Van Waes *et al* (2006) showed that CK2 contributes to the activation of IKK and NF-κB in response to serum factors, which suggests that CK2 and IKK2 are key candidates for targeting the NF-κB pathway in head and neck squamous cell carcinoma (HNSCC). NF-κB activation has been broadly shown and associated with progression in intraepithelial

premalignant and malignant squamous neoplasms of the head and neck as well as uterine cervix.[43]

Christine H. Chung, Joel S. Parker, Kim Ely, Jesse Carter *et al* (2006) studied the high- risk signature for disease recurrence using formalin-fixed tissues of HNSCC tumors and compared the results with an independent data set obtained from fresh frozen tumors. Gene expression was determined in 40 samples, including 34 formalin-fixed tissues and 6 matched frozen tissues, from 29 HNSCC patients. A 75-gene list predictive of disease recurrence was determined by training on the formalin- fixed tumor data set and tested on data from the independent frozen tumor set from 60 HNSCC patients. They concluded that global gene expression analysis is feasible using formalin fixed tissue. The 75-gene list can be used a prognostic biomarker of recurrence and our data suggest that the molecular determinants of EMT and NF-κB activation can be targeted as the novel therapy in the identified high-risk patients.[46]

Tin Lap Lee, Xin Ping Yang, Bin Yan, Jay Friedman *et al* (2007) studied gene signatures that was differentially expressed in head and neck squamous cell carcinomas (HNSCC) that are related to alterations in transcription factor-κB (NF-κB) and TP53 previously associated with decreased cell death, response to therapy and worse prognosis. Unique gene signatures expressed by HNSCC lines were identified by cDNA microarray, principal components, and cluster analyses and validated by quantitative reverse

transcription-PCR (RT-PCR) and *in situ* hybridization. Bioinformatic analysis of the promoters and ontogeny of these cluster genes was done. NF-κB promotes expression of a novel NF-κB – related gene signature and cell survival in HNSCC that weakly express TP53, a subset previously associated with inactivated wild-type TP53, greater resistance to chemoradiotherapy, and worse prognosis.[45]

Zhong Chen, Bin Yan, Carter Van Waes *et al* (2008) used immunohistochemistry (IHC), Reverse-phase protein microarray (RPMA), Western blot analysis and Enzyme linked Immunosorbant Assay (ELISA) to determine which pathways are activated and how they correlate with prognosis, select therapy targeting these pathways and to use changes in phosphorylation, expression of protein and functional changes such as TUNEL or caspase cleavage together as early biomarkers of response to the treatment.

Serum cytokines regulated by NF-κB represent biomarkers of response, recurrence and survival in HNSCC.[40]

NF-κB AND LEUKOPLAKIA

Squamous cell carcinoma is the most common malignancy of the oral cavity which is often preceded by premalignant lesions, the most common of which is leukoplakia.[60] Epithelial malignancies of the oral cavity often begin as preneoplastic lesions in the form of inflammatory lesions such as leukoplakia. Leukoplakia is associated with tobacco and alcohol use and chronic inflammation with a

higher risk of malignant transformation to oral squamous cell carcinoma.[63]

Nuclear factor kappa B has been implicated in the development of head and neck squamous cell carcinoma from premalignancy, progression to invasion, metastasis and treatment resistance.[51] Transcription factor NF-κ B is an early response gene that is found to be elevated in subjects with tobacco use, chronic inflammatory conditions of oropharynx and head and neck cancer. Inflammatory gene expressions are induced by the downstream of cytokine and growth factor receptors, such as signal transducers and activators of transcription. The expression of NF-κ B has been found to be gradually increasing from premalignant lesions to invasive cancer.[62]

NF-κB AS A POTENTIAL MOLECULAR TARGET FOR CANCER THERAPY

NF-κB is constitutively activated in a large number of epithelial and hematologic malignancies, strongly suggest that NF-κB inhibitors would be useful in cancer therapy. Inhibition of NF-κB has been found to be an important mechanism of action of steroids, nonsteroidal anti-inflammatory drugs (NSAID) and natural and synthetic compounds that show therapeutic and preventive activity with acceptable safety profiles.[49]

NSAIDS AND CANCER PREVENTION

NSAIDs such as aspirin, sulindac, ibuprofen, celecoxib, have been shown to inhibit NF-κB activation and arachidonic acid

inflammatory pathways upstream and downstream of NF-κB. Strategy for blocking NF-κB include an upstream strategy and a NF-κB targeting strategy.[50] Blocking the activation of NF-κB signaling pathway using:

a) proteasome inhibitors (such as PS-341, MG 132);
b) IKK inhibitors (such as NSAIDs, sulfa salazine, arsenic trioxide, curcumin, thlidomide);
c) cell-permeable peptides (such as SN-50);
d) antioxidants (such as disulfiram, glutathione); or
e) the recombinant adenovirus-mediated overexpression of the IκBα gene.

On the other hand, the NF-κB targeting strategy includes:

(a) blocking the DNA binding of NF-κB using decoy oligodeoxynucleotides (ODNs)
(b) blocking the transactivation of NF-κB using glucocorticoids
(c) interfering with mRNA using NF-κB antisense oligonucleotide (ASO)[51,52]

CORTICOSTEROIDS AND CYTOTOXIC AGENTS USED FOR THERAPY

The cytotoxic effects of corticosteroids in combination with other DNA-damaging agents led to the use of steroid-based regimens as a current mainstay of treatment of certain leukemias, lymphomas and myelomas. Subsequently, corticosteroids were shown to mediate many of their anti-inflammatory and tumor cytotoxic effects through inhibition of NF-κB, and these lymphoid

malignancies and supporting host responses were found to be exquisitely dependent on NF-κB regulated survival or inflammatory mechanisms.[53,54]

PROTEASOME INHIBITORS

A new class of therapeutic agents under development are proteasome inhibitors, which regulate degradation of IκB and inhibit NF-κB, as well as turnover of other cellular proteins.[55,56]

IKK INHIBITORS

IKKβ antagonists that more specifically inhibit IκB and the NF-κB classic pathway initially implicated in cancers have been the subject of intensive development and preclinical studies.[57,58]

PATIENT CHARACTERISTICS:

Forty cases of OSCC (Group I), twenty cases of Oral Leukoplakia (Group II) and Twenty cases of normal mucosa (Group III) were analyzed for immunoreactivity of NF-κ B protein. All the samples in group I, II and III were taken from the buccal mucosa.

The patient's age ranged from 35 to 92 years (mean 57.5 ± 13.3) in group I from 26 to 59 years (mean 42.2 ± 10.1) in group II and from 20 to 42 years (mean 27.9 ± 4.8) in group III **(Table 1, Graph 1)**

Of the forty patients in group I, 55% (n=22) were men and 45% (n=18) were women. In group II all the cases were men, whereas in group III, 70% (n= 14) were men and 30% (n=6) were women **(Table 2, Graph 2)**

Depending upon habits, patients were segregated into Areca Chewers, Tobacco Chewers, Areca & Tobacco Chewers and Smokers & Chewers. In group I, 13% (n=5) of patients were Areca Chewers, 43% (n=17) of patients were Tobacco Chewers, and 28% (n=11) were Areca & Tobacco Chewers, 3% (n=1) were both Smokers & Chewers and 15% (n=6) had no habits. Entire group III had no smoking or chewing habits **(Table 3, Graph 3)**

According to the histopathological grading in OSCC, 23% (n=18) of patients had well differentiated OSCC, 24% (n=19) of patients had moderately differentiated OSCC and 11% (n=9) of patients had poorly differentiated.

Distribution of NF-κ B Staining Among 3 Groups:

NF-κ B revealed positivity in group I (Figure 7 & 8), group II (Figure 11& 12) and group III (Figure 15 & 16). In Group II positive staining was observed in 100 % of the cases, whereas in Group I and Group III, positive staining was observed in 97% and 80% respectively.

Tissue localization of stain (Table 4, Graph 4)

NF – κB staining was seen both in the epithelium as well as in the connective tissue. In connective tissue the staining was seen in lymphocytes, muscle fibres and endothelial cells.

In group I, 65% (n=26) of cases showed nuclear staining in the epithelium and connective tissue, whereas in 35% (n=14) of cases showed cytoplasmic staining, In groupII 80% (n=16) showed nuclear staining while 20% (n=4) showed cytoplasmic staining and in group III 35% (n=7) showed nuclear staining and 65% (n=13) cases showed cytoplasmic staining. In connective tissue, staining was predominantly seen in lymphocytes, muscle fibres and endothelial cells. These results were statistically significant (p=0.011)

INTENSITY OF STAIN

Distribution of staining intensity in the basal layers among the groups (Table 5, Graph5)

In the basal layer, mild intensity of staining was seen in 18% (n=7) of OSCC, 20% (n=4) of leukoplakia and 15% (n=3) of normal cases. Moderate staining was seen in 45% (n=18) of OSCC , 45% (n=9) of leukoplakia and 30% (n=6) of normal cases whereas 35% (n=14) of OSCC, 35% (n=7) of leukoplakia and 35% (n=7) of normal cases showed intense staining.

Distribution of staining intensity in the suprabasal layers among the groups (Table 6, Graph 6)

In the suprabasal layer, mild intensity of staining was seen in 18% (n=7) of OSCC, 5% (n=1) of leukoplakia and 5% (n=1) of normal cases. Moderate staining was seen in 58% (n=23) of OSCC, 70% (n=14) of leukoplakia and 65% (n=13) of normal cases whereas 23% (n=9) of OSCC, 25% (n=5) of leukoplakia and 10% (n=2) of normal cases showed intense staining. The results were statistically significant (p=0.048)

Comparison of staining intensity in the connective tissue among the groups (Table 7, Graph 7)

In 40 cases of OSCC 23% (n=9) and in normal 50% (n=10) showed no stain. Mild intensity of staining was seen in 18% (n=7) of OSCC,10% (n=2) of both leukoplakia and normal cases. Moderate intensity of staining was seen in 35% (n=14) of OSCC and 70% (n=14) of leukoplakia and 25% (n=5) of normal cases. And

intense staining was seen in 25% (n=10) of OSCC and 20% (n=4) of leukoplakia and 15% (n=3) of normal cases. The results were statistically significant (p=0.007)

Comparison of staining intensity in the epithelium and connective tissue among the groups (Table 8, Graph 8)

In 40 cases of OSCC 11.1% (n=1) and in 20 cases of normal 40% (n=4) showed no stain both in the epithelium and in the connective tissue. Mild staining in the epithelium and no stain in the connective tissue was seen in 11.1% (n=1) cases of OSCC. Moderate staining in the epithelium and no stain in the connective tissue was seen in 66.7% (n=6) of OSCC and 60% (n=6) of normal cases. Moderate staining in the epithelium and mild staining in the connective tissue was seen in 100% (n=7) of OSCC, 100% (n=2) of leukoplakia and 50% (n=1) of normal cases. Moderate staining in the epithelium and intense staining in the connective tissue was seen in 100% (n=10) of leukoplakia and 33.3% (n=1) of normal cases. Intense staining in the epithelium and no stain in the connective tissue was seen in 11.1% (n=1) of OSCC. Intense staining in the epithelium and mild staining in the connective tissue was seen in 50% (n=1) of normal cases. Intense staining in the epithelium and moderate staining in the connective tissue was seen in 42.9% (n=6) of both OSCC and leukoplakia, 60% (n=3) of normal. Intense staining in both the epithelium and connective tissue was seen in 100% (n=10) of OSCC and 66.7% (n=2) of normal. Staining intensity in the epithelium and connective tissue of

OSCC and leukoplakia were statistically significant and they were 0.001 and 0.043 respectively.

Comparison of mean labeling index among the study groups

In OSCC, 26/40 cases showed nuclear staining and their mean labeling index was 25.96. In leukoplakia, 16/20 cases showed nuclear staining which had the mean labeling index as follows 20.84. In normal, 7/20 cases showed nuclear staining and its mean labeling index is 30.93, but this difference was not statistically significant (p=0.262).

Comparison of mean labeling index between OSCC and normal, Leukoplakia and normal & OSCC and leukoplakia

Comparison of mean labeling index between OSCC (54.34) and normal (44.97) was done. Comparison of mean labeling index between leukoplakia (51.17) and normal (44.97) was done. Similarly the mean labeling index of OSCC (54.34) was compared to leukoplakia (51.17), but these differences were not statistically significant.

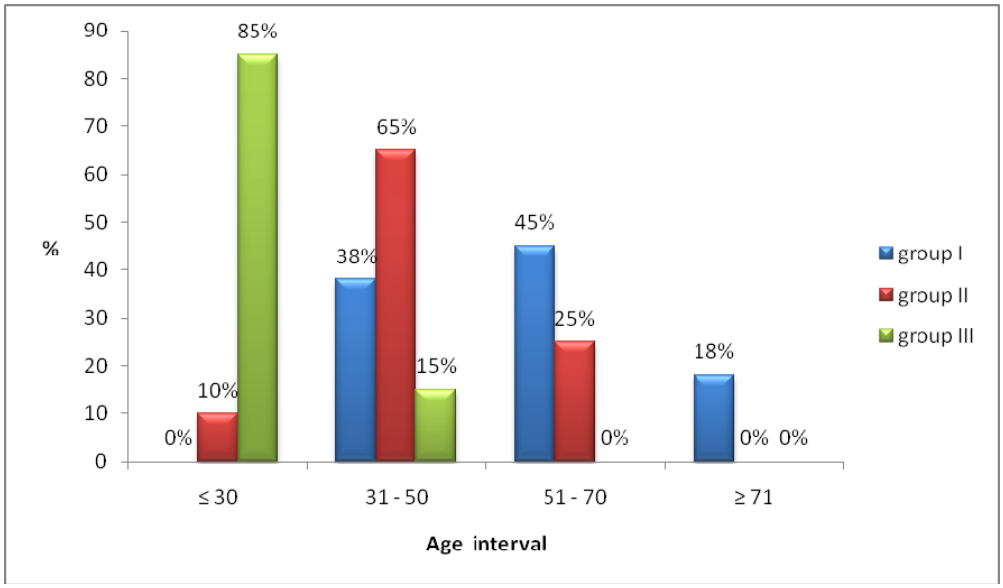
The inter-observer agreement for the intensity of stain for all the 3 groups was arrived at kappa values of 0.648

TABLE 1: AGE OF THE SUBJECTS IN THE STUDY GROUPS

Age	Group I		Group II		Group III		p value
	n	%	n	%	n	%	
≤30	0	0	2	10	17	85	0.000*
31-50	15	38	13	65	3	15	
51-70	18	45	5	25	0	0	
≥71	7	18	0	0	0	0	

*p value ≤0.05 was considered to be statistically significant

GRAPH 1: AGE OF THE SUBJECTS IN THE STUDY GROUPS



Group I – OSCC

Group II – Leukoplakia

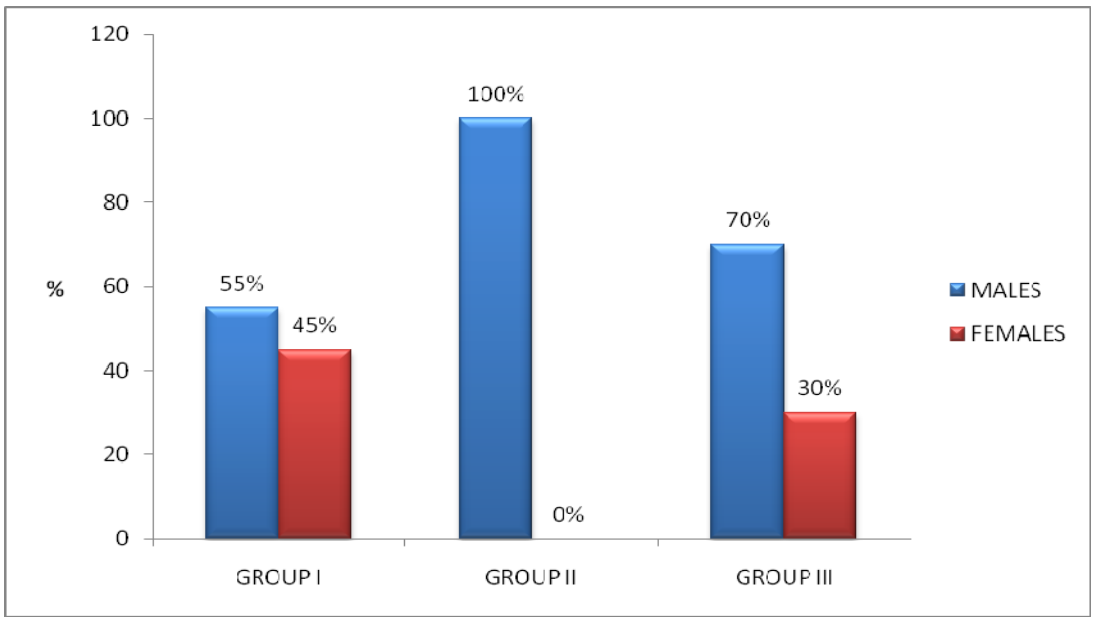
Group III – Normal

TABLE 2: DISTRIBUTION OF GENDER IN THE STUDY GROUPS

CASES	MALE		FEMALE		p value
	n	%	n	%	
Group I	22	55	18	45	0.002*
Group II	20	100	0	0	
Group III	14	70	6	30	

*p value ≤ 0.05 was considered to be statistically significant

GRAPH 2: DISTRIBUTION OF GENDER IN THE STUDY GROUPS



Group I – OSCC

Group II – Leukoplakia

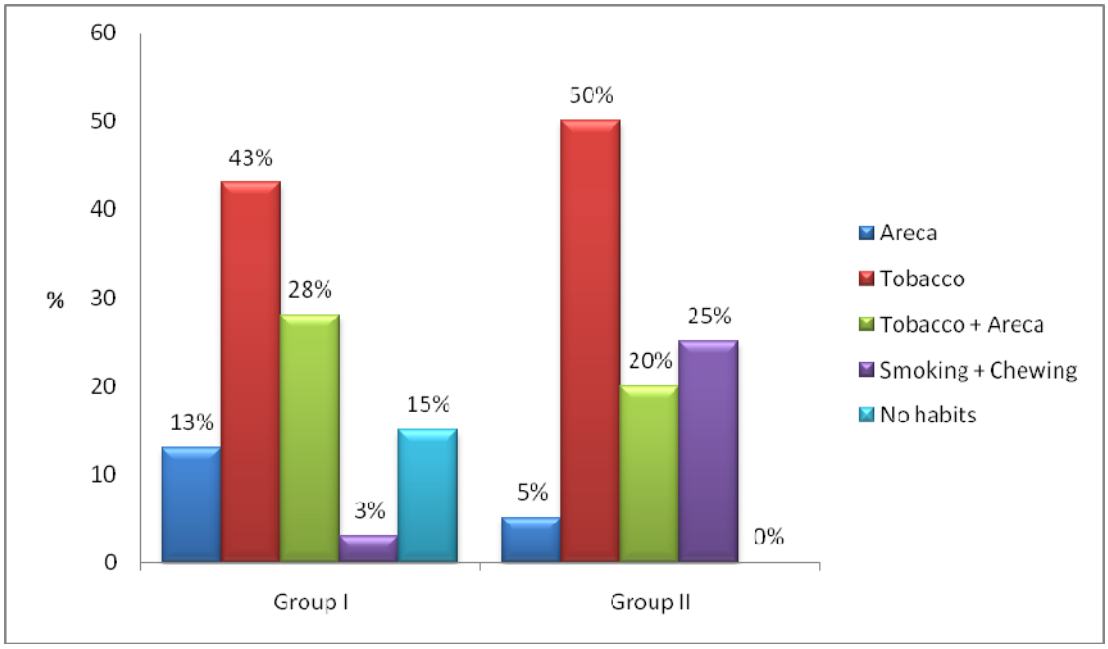
Group III – Normal

TABLE 3: DISTRIBUTION OF HABITS IN THE STUDY GROUPS

Habits	Group I		Group II		p value
	n	%	n	%	
Areca Chewers	5	13	1	5	0.000*
Tobacco Chewers	17	43	10	50	
Tobacco + Areca	11	28	4	20	
Smoking + Chewing	1	3	5	25	
No habits	6	15	0	0	

*p value ≤ 0.05 was considered to be statistically significant

GRAPH 3: DISTRIBUTION OF HABITS IN THE STUDY GROUPS



Group I – OSCC

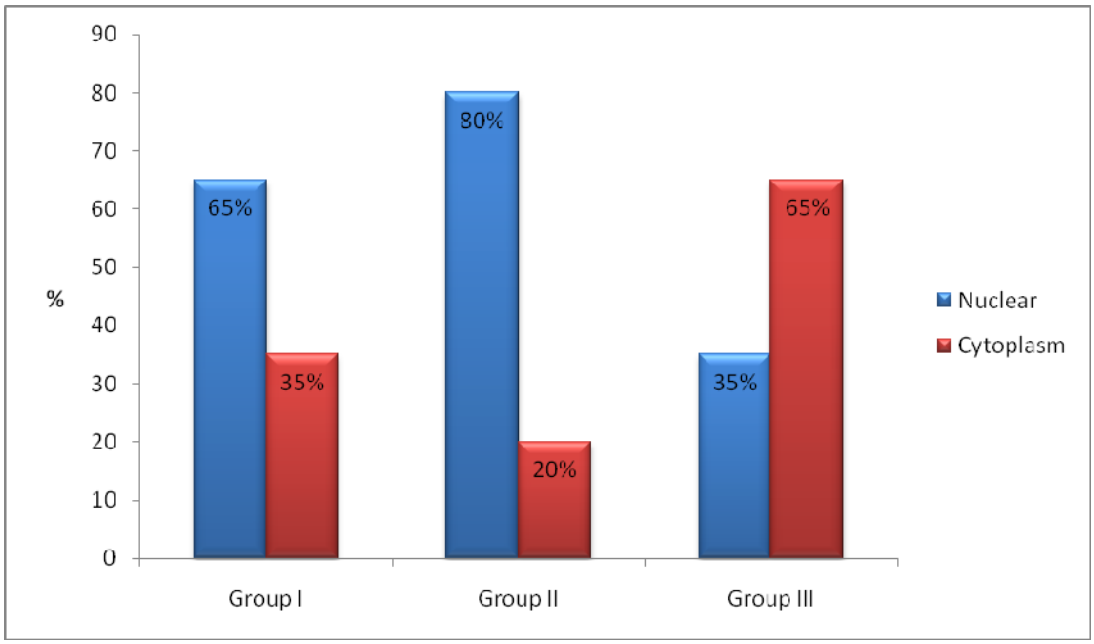
Group II - Leukoplakia

TABLE 4: LOCALIZATION OF NF-κ B STAINING AMONG THE GROUPS

	GROUP I (%)	GROUP II (%)	GROUP III (%)	p value
NUCLEAR	65	80	35	0.011*
CYTOPLASMIC	35	20	65	

*p value ≤ 0.05 was considered to be statistically significant

GRAPH 4: LOCALIZATION OF NF-κ B STAINING AMONG THE GROUPS



Group I – OSCC

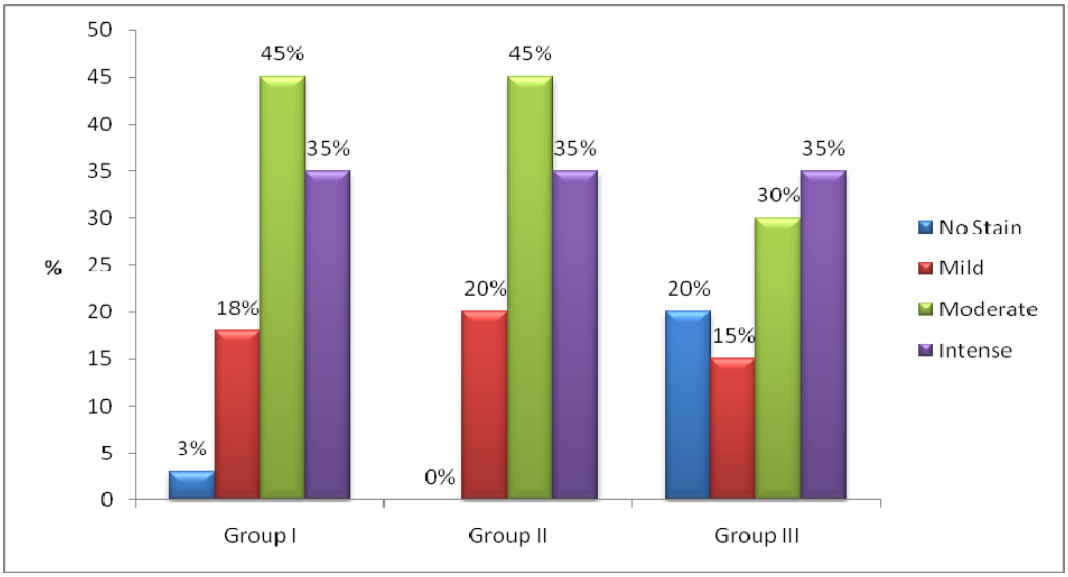
Group II – Leukoplakia

Group III – Normal

TABLE 5: DISTRIBUTION OF STAINING INTENSITY IN THE BASAL LAYER AMONG THE GROUPS

Intensity	Group I		Group II		Group III		p value
	n	%	n	%	n	%	
No Stain	1	3	0	0	4	20	0.165
Mild	7	18	4	20	3	15	
Moderate	18	45	9	45	6	30	
Intense	14	35	7	35	7	35	

GRAPH 5: DISTRIBUTION OF STAINING INTENSITY IN THE BASAL LAYER AMONG THE GROUPS



Group I – OSCC

Group II – Leukoplakia

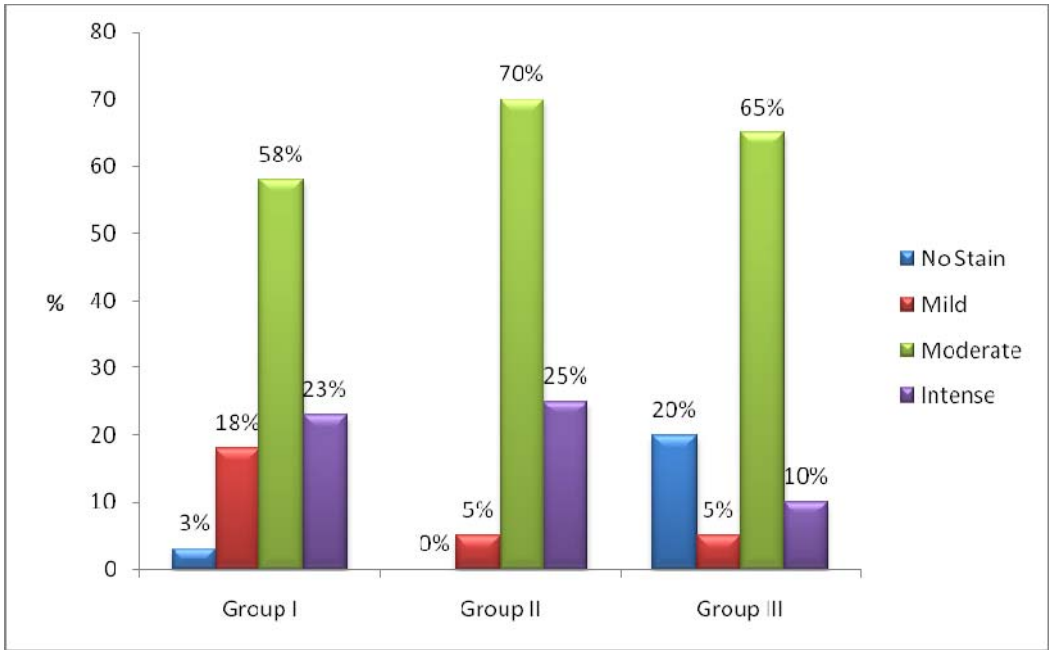
Group III – Normal

TABLE 6: DISTRIBUTION OF STAINING IN THE SUPRABASAL LAYER AMONG THE GROUPS

Intensity	Group I		Group II		Group III		p value
	n	%	n	%	n	%	
No Stain	1	3	0	0	4	20	0.048*
Mild	7	18	1	5	1	5	
Moderate	23	58	14	70	13	65	
Intense	9	23	5	25	2	10	

*p value ≤ 0.05 was considered to be statistically significant

GRAPH 6: DISTRIBUTION OF STAINING IN THE SUPRABASAL LAYER AMONG THE GROUPS



Group I – OSCC

Group II – Leukoplakia

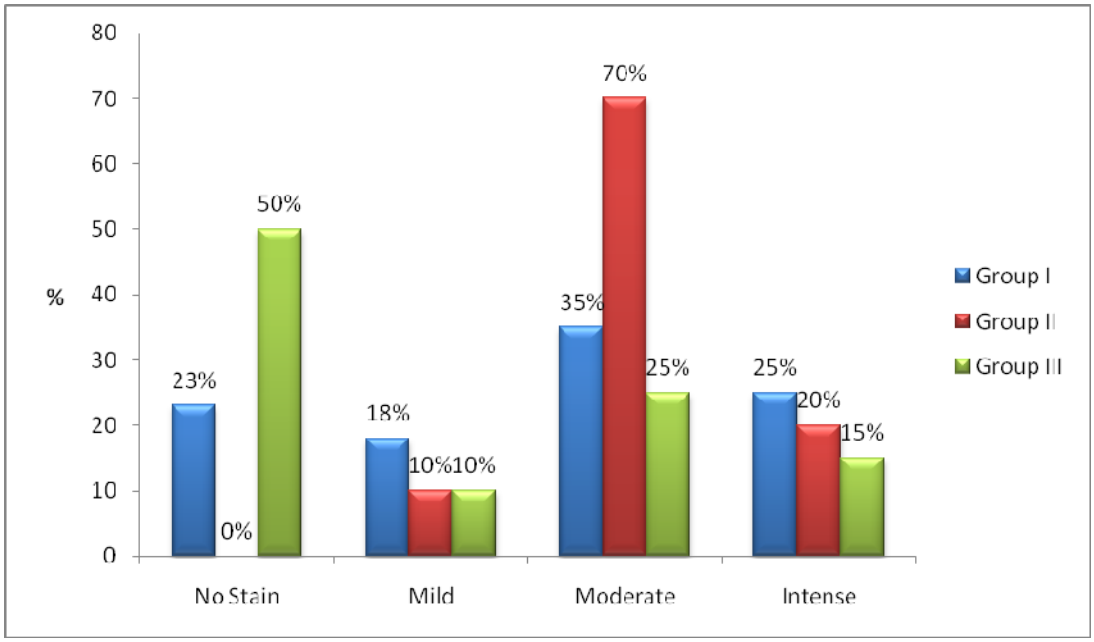
Group III – Normal

TABLE 7: DISTRIBUTION OF STAINING INTENSITY IN THE CONNECTIVE TISSUE AMONG THE GROUPS

Intensity	Group I		Group II		Group III		p value
	n	%	n	%	n	%	
No Stain	9	23	0	0	10	50	0.007*
Mild	7	18	2	10	2	10	
Moderate	14	35	14	70	5	25	
Intense	10	25	4	20	3	15	

*p value ≤ 0.05 was considered to be statistically significant

GRAPH 7: DISTRIBUTION OF STAINING INTENSITY IN THE CONNECTIVE TISSUE AMONG THE GROUPS



Group I – OSCC

Group II – Leukoplakia

Group III – Normal

TABLE 8: COMPARISON OF STAINING INTENSITY IN THE EPITHELIUM AND CONNECTIVE TISSUE WITHIN THE GROUPS

EPITHELIUM			CONNECTIVE TISSUE								p value
			No stain		Mild		Moderate		Intense		
			n	%	n	%	n	%	n	%	
	GROUP I	No stain	1	11.1	0	0	0	0	0	0	0.001*
		Mild	1	11.1	0	0	0	0	0	0	
		Moderate	6	66.7	7	100	8	57.1	0	0	
		Intense	1	11.1	0	0	6	42.9	10	100	
	GROUP II	Moderate	-	-	2	100	8	57.1	10	100	0.043*
		Intense	-	-	0	0	6	42.9	0	0	
	GROUP III	No stain	4	40	0	0	0	0	0	0	0.102
		Moderate	6	60	1	50	2	40	1	33.3	
		Intense	0	0	1	50	3	60	2	66.7	

*p value ≤ 0.05 was considered to be statistically significant

GRAPH 8: COMPARISON OF STAINING INTENSITY IN THE EPITHELIUM AND CONNECTIVE TISSUE WITHIN THE GROUPS

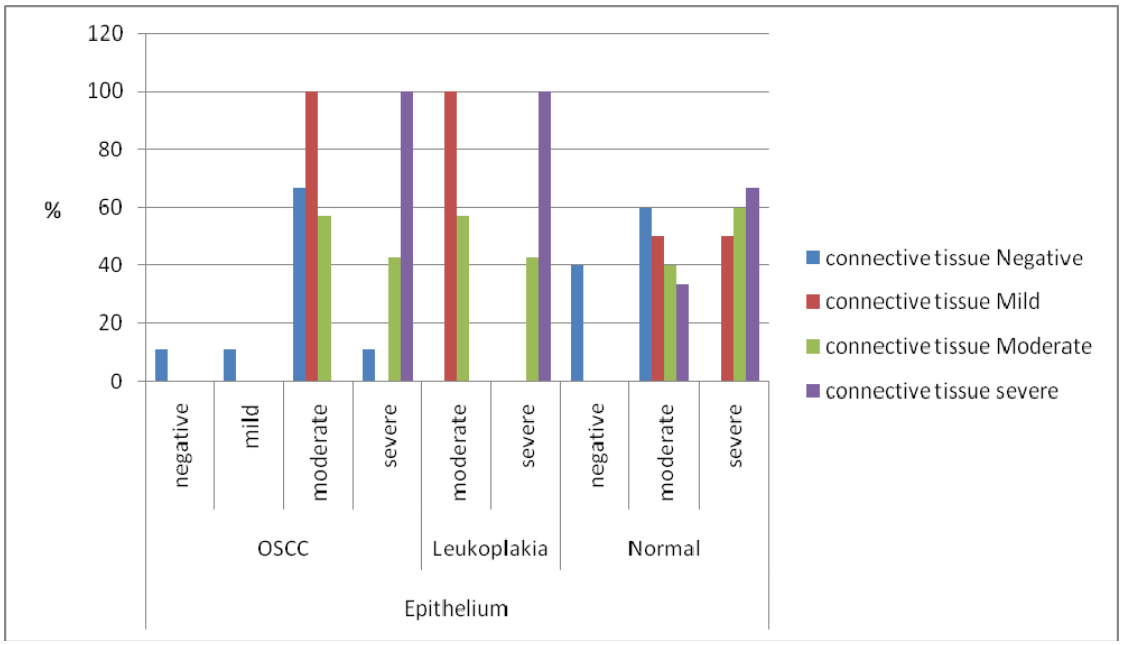


Figure 1: Armamentarium

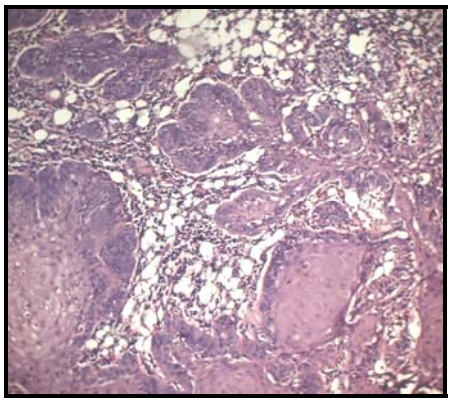
Figure 2: Antibodies

Figure 3: Oral squamous cell carcinoma

Figure 4: Oral leukoplakia

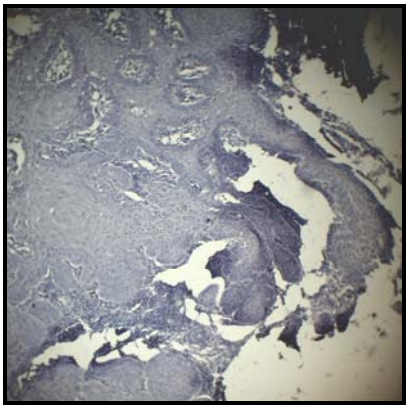
Oral squamous cell carcinoma

Figure 5:



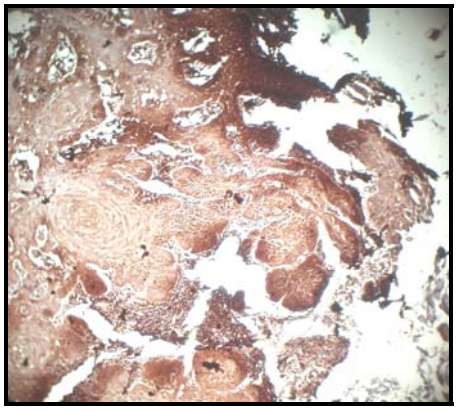
H & E stain; 10x

Figure 6:



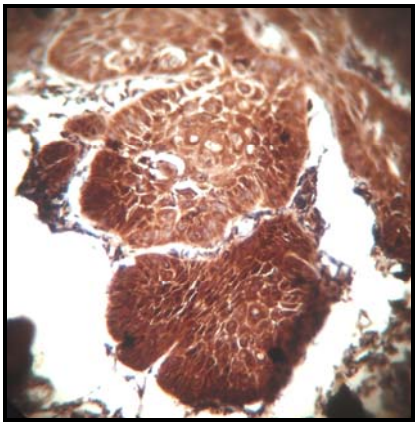
Negative NF- κ B stain; 10x

Figure 7:



Positive NF- κ B stain; 10x

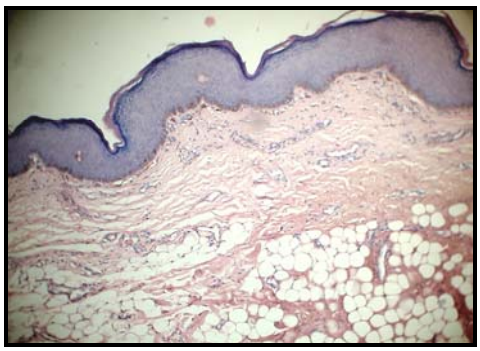
Figure 8:



Positive NF- κ B stain; 40x

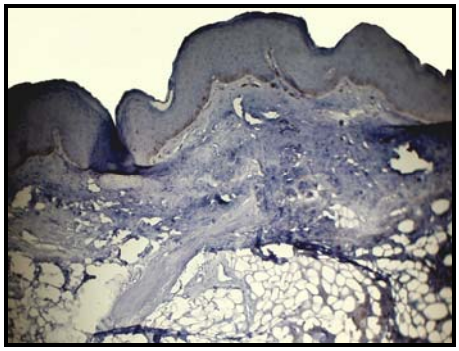
Oral Leukoplakia

Figure 9:



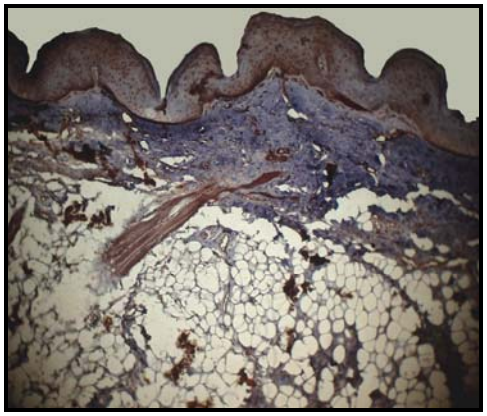
H & E stain; 10x

Figure 10:



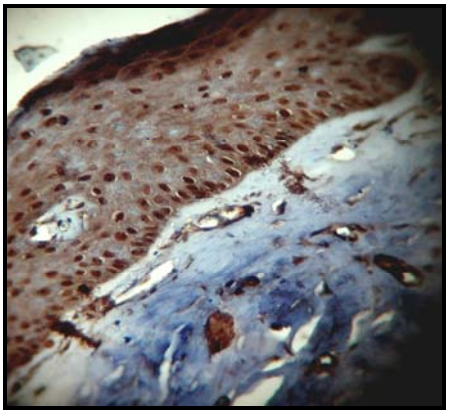
Negative NF- κ B stain; 10x

Figure 11:



Positive NF- κ B stain; 10x

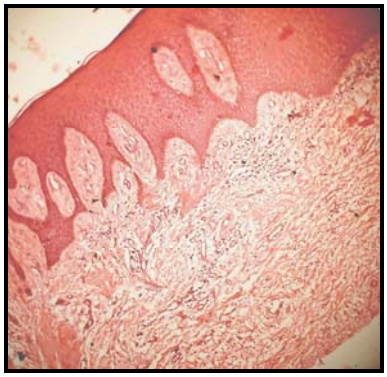
Figure 12:



Positive NF- κ B stain; 40x

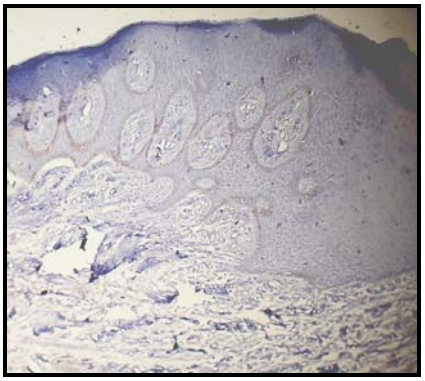
Normal Oral Mucosa

Figure 13:



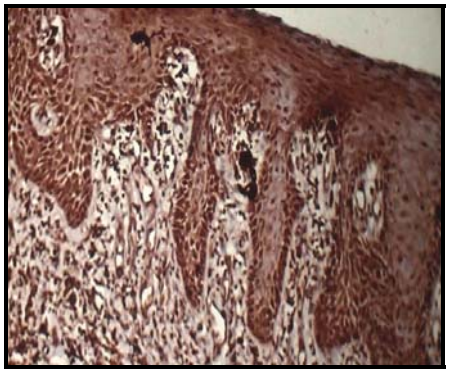
H & E stain; 10x

Figure 14:



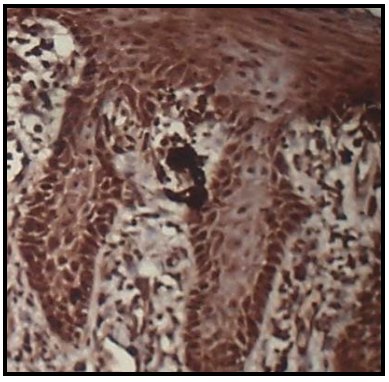
Negative NF- κ B stain; 10x

Figure 15:



Positive NF- κ B stain; 10x

Figure 16:



Positive NF- κ B stain; 40x

Nuclear factor κ B (NF-κ B) is a transcription factor that induces the expression of various genes, that influence inflammatory reactions, embryonic morphogenesis and antiapoptosis. Intracellular signaling mechanism of NF-κ B activation is effected by stimulators such as tumor necrosis factor α (TNF-α) and many other signals.[44] An inhibitor of NF-κ B known as Iκ B, which holds NF-κ B in the cytoplasm gets phosphorylated and ubiquitinated by ubiquitin ligase and degraded by 26S proteosome and active NF-κ B translocates from the cytoplasm into the nucleus and binds with the specific sequence in the promoter of target genes.[37] These genes include pro-inflammatory cytokines, chemokines and cell survival genes. Activation of NF-κ B plays a key role by which cells are resistant to TNF- mediated apoptosis, thus being associated with development and progression and metastasis of several malignancies including squamous cell carcinoma.[39]

Oral dysplasia is often a common precursor of oral cancer. Progression to cancer varies widely ranging from 5% to 20%. The assessment and severity of dysplasia is based on architectural disturbances accompanied by cytological atypia which are objective in nature. Since NF – κB is a transcription factor that is known to involve in growth, invasion and anti-apoptotic activity of cancer cells. In this study we wanted to study expression in OSCC and leukoplakia and compare its activity with that of normal oral

mucosa to ascertain if it could be used as a surrogate marker for malignant transformation.[8]

PATIENT CHARACTERISTICS

In our study the mean age in OSCC cases was 57.5 ± 13.3 years. **Mishra *et al,*** studied 66 cases of OSCC and reported the mean age in their study group as 52.9 ± 10.8 years. In the study done by **Nakayama *et al,*** the mean age was 70.2 years in 36 cases and **Yu- Chao chang *et al*** reported age range from 50-59 years with a mean age of 55 years in 442 cases of OSCC. In a study by **Ranganathan *et al***, the age ranged from 56-85 years with a mean age of 71.2 years in 10 cases.

In our study, the age presentation for leukoplakia ranged from 26 to 59 years with a mean of 42.2 ± 10.1 years which is consistent with the study by **Thavaraj *et al*** with a mean age of 36.67years in 500 patients. **Yu- Chao chang *et al*** reported an age range from 20-29 years and with a mean age of 25 years in 344 cases of oral leukoplakia. In the study by **Lumerman *et al*** the age range was 25 to 92 years with a mean age of 59.3 years.

In our study subjects who had OSCC were predominantly males and they constituted to about 70%. The male to female ratio was 2.2:1 and this finding was consistent with that of **Napier *et al*** where the male: female ratio was 1.9:1 and the male: female ratio was 3.9:1 with that of **Mishra *et al***.

In our study all the patients who had leukoplakia were males which is similar to a male: female ratio of 0.7 : 0.4 reported by

Saraswathi *et al* in their study of 2017 patients at our centre earlier. In a study by **Thavaraj *et al*** the male: female ratio was 9.8:1 They also stated that number of males was around five times more than the females.

In this study, those patients who presented with OSCC, had the habit of chewing tobacco 17% and 5% of the patient had the habit of only Areca and about 11% of patients used both areca and tobacco . This finding is consistent with that of **Thavaraj *et al*** who reported that the majority of patients with OSCC in their study about 9.8% had the habit of chewing tobacco whereas 5.6% patients had the habit of only areca.

In this study, of 20 patients who presented with leukoplakia, 10% had the habit of chewing tobacco and 4% had the habit of both areca nut and tobacco chewing. These findings are consistent with that of **Saraswathi *et al*** who reported that 10% of the patients had the habit of chewing areca nut and 8% had the habit of betel quid chewing.

STAINING CHARACTERISTICS OF NF – κB

In our study, cytoplasmic expression of NF – κB was seen in 77.5% (n=31) of OSCC. When we analysed the pattern of cytoplasmic staining, we observed that 17.5% (n=7) of the cases showed mild intensity of staining, while 35% (n=14) showed moderate and 25% (n=10) showed intense staining. Nuclear expression of NF – κB was seen in 65% (n=26) 0f cases. The

nuclear percentage positivity increased with increasing grade of OSCC.

Dysregulation of NF – κB expression and its activation is frequently observed in many human cancers. Activated NF – κB shows nuclear expression and it indicates an early event in carcinogenesis. Once activated, it controls the expression of several genes that regulate cell cycle (cyclin D1), differentiation (p^{21}), cell survival (Bcl – 2), growth factors (VEGF) cell adhesion (VCAM, ECAM) and angiogenesis (MMPs).

Our results were consistent with **Nakayama *et al*** who also reported 78.2% cytoplasmic expression in 36 cases of OSCC. There are other studies which states 64% of cytoplasmic expression as observed by **Asha Nair *et al.*** The explanation for the absence of cytoplasmic expression of NF – κB in OSCC has not been indicated in this study.

In all these studies there was an association between tumor progression and extent of nuclear translocation of NF – κB. In our study also the moderately differentiated OSCC exhibited increased nuclear expression which is consistent with tumor progression. **Nakayama *et al*** suggested that high expression level of NF – κB contributed to the malignant behavior and anti apoptotic activity of OSCC. **Mishra *et al*** reports that p50 homodimers transcriptionally regulate anti – apopotic Bcl -2 which is shown to be over expressed in oral cancer cells and inhibits terminal differentiation of oral keratinocyte. The exact mechanism by which NF – κB is

constitutively activated in OSCC though not fully understood, it has been suggested that autocrine expression of interleukins and EGFR may play an important role in the activation of NF – κB.

In inflammation associated cancer, non-genetic stimuli encourage the survival and proliferation of cells. NF – κB has dual actions in tumor promotion; first by preventing the death of cells with malignant potential and second by stimulating the production of pro-inflammatory cytokines in inflammatory cells in tumor mass. These cytokines then signals the cell to promote their survival and proliferation. Classical NF – κB pathway i.e, the IKK – β depedent NF – κB activation pathway might show the molecular link between inflammation and tumor promotion[48,49]. So, in chronic inflammatory diseases, pro-inflammatory factors cause accumulation of DNA damage in dormant pre-malignant cells in tumor microenvironment to become malignant.[44]

In our study in leukoplakia, cytoplasmic expression of NF – κB was seen in 100% (n=20) of the cases. When we analysed the pattern of staining, mild intensity of staining was observed in 10% (n=2) of case, 70% (n=14) showed moderate intensity, and 20% (n=4) showed intense cytoplasmic expression. Our study also showed nuclear expression of NF – κB in 80% (n=16) of cases. When we further analysed in order to understand the intensity of staining we observed that proliferating cells showed more staining than differentiated cells. We observed that nuclear expression is

seen in those cases of leukoplakia which expressed histologically dysplastic alterations and an increase in inflammatory response.

Nayakama *et al* observed 4% moderate staining and 11% of mild staining of NF – κB expression in the epithelium of 15 dysplasia (leukoplakia) samples. They also reported that staining was seen in both basal and spinous epithelium cells and staining of NF – κB was seen in the basal epithelial cells of epithelial dysplasia (leukoplakia).

They suggest that NF – κB is an important mediator in chronic inflammatory process. Aberrant and persistent tissue inflammation is believed to play an important role in the occurence of tissue fibrosis and cancer. One of the etiological factors of leukoplakia is associated with area nut chewing habit. They propose that areca nut extract was found to activate NF – κB in human oral keratinocytes and one of the pathogenic mechanisms of leukoplakia may be due to increased expression of NF – κB in response to areca nut. NF – κB activates cytokines such as IL – 1 and TN F – α which can result in amplification of inflammatory, response and persistence of chronic inflammation at local site.[61]

So, in leukoplakia we observed nuclear staining in 16% of the cases. This nuclear expression of NF – κB by the epithelial cells is correlated with the amount of cytotoxic cell infiltration suggesting that increased NF – κB activity may represent the basis of maintenance of the inflammatory response. This can lead to the expression of genes that are mediated in carcinogens. So, expression

of nuclear staining in leukoplakia can be used as an indicator for carcinogenesis.

The nuclear expression in our cases showed histopathologically a higher number of inflammatory cells which could account for its expressivity. We would like to corroborate our finding by studying more cases.

Compared to the normal cases the cytoplasmic intensity and nuclear immunoreactivity in OSCC and leukoplakia was increased and also we could find a statistically significant association between the two patterns of staining.

- The study group comprised of Group I (OSCC n=40), Group II (leukoplakia n=20) and Group III (Normal n=20).
- In OSCC, there was 98% positivity for NF – κB with 35% of cytoplasmic expression and 65% of nuclear expression.
- In leukoplakia; there was 100% positivity for NF – κB expression; with 20% of cytoplasmic expression and 80% of nuclear staining.
- In normal, there was 80% of positivity for NF – κB expression within 65% of cytoplasmic expression and 35% of nuclear expression.
- In OSCC and leukoplakia when the epithelial staining intensity was compared with the connective tissue staining intensity there was a statistically significant correlation (p=0.001) and (p=0.043) respectively.
- Nuclear expression of NF – κB exhibited difference (p=0.011) between the groups.

In conclusion, our results show that there is increased expression of NF – κ B in OSCC and leukoplakia when compared to normal. Although the mean labeling index did not show any significant difference between OSCC and leukoplakia, further studies on a larger sample will help in ascertaining the exact role of NF – κ B expression in leukoplakia samples.

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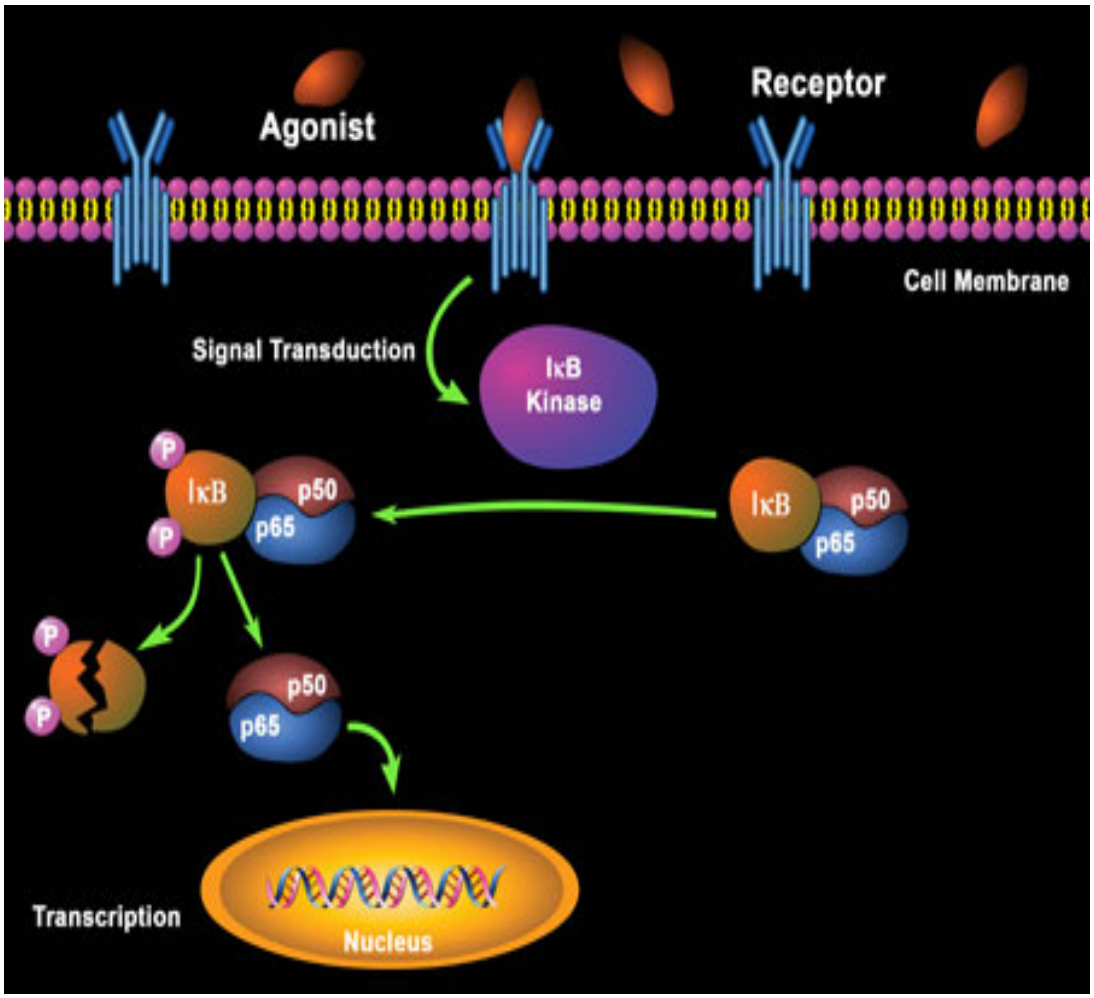
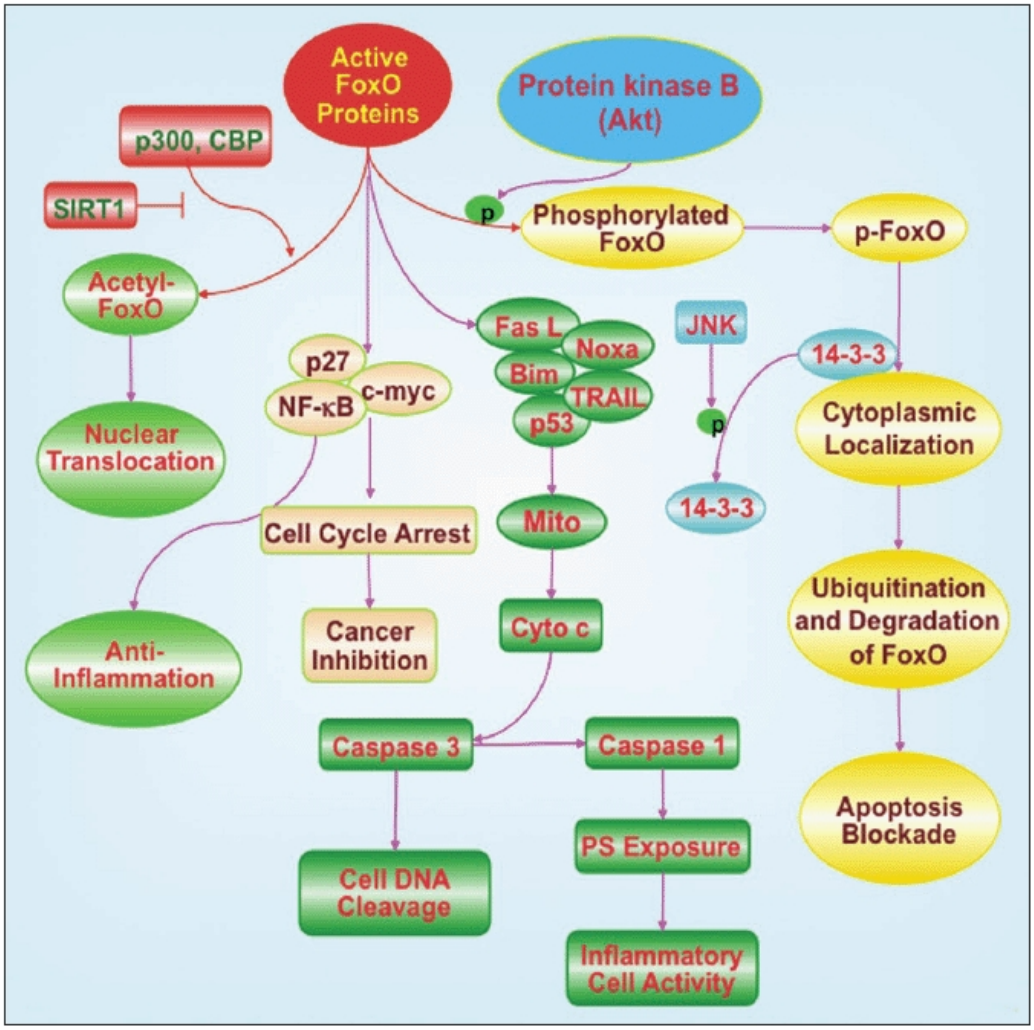
PATHWAY OF NUCLEAR FACTOR-κ B



Courtesy – Gilmore TD, Introduction to NF-κB: players, pathways, perspectives, Oncogene, 2006, 25:6680-6684.

ACTIVATION OF NF- κ B AND GENES INVOLVED



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ABBREVIATIONS

1. RHD	**-**	**Rel Homology Domain**
2. IκB	**-**	**Inhibitor of NF-κB**
3. IKK	**-**	**IκB Kinase**
4. NEMO	**-**	**NF-κB Essential Modulator**
5. NIK	**-**	**NF-κB inducing Kinase**
6. VCAM	**-**	**Vascular Cell Adhesion Molecule**
7. ICAM	**-**	**Intercellular Cell Adhesion Molecule**
8. TRAF	**-**	**TNF – Receptor Associated Factor**
9. IAP	**-**	**Inhibitor of Apoptosis Protein**
10. JNK	**-**	**Jun N-terminal Kinase**
11. TNF	**-**	**Tumor Necrosis Factor**
12. IL	**-**	**Interleukins**
13. COX	**-**	**Cyclo oxygenase**
14. INOS	**-**	**Inducible Nitric Oxide Synthase**
15. MMP	**-**	**Matrix Metallo Proteinase**
16. uPA	**-**	**Serine protease urokinase – type plasminogen activator**